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Folate Deficiency in Rats Bearing the Walker Tumor 256 and the Novikoff Hepatoma[1]

Lionel A. Poirier[2]

Carcinogen Metabolism and Toxicology Branch, National Cancer Institute, Bethesda, Maryland 20014

SUMMARY

A folate deficiency was noted in rats bearing the Novikoff hepatoma and the Walker 256 carcinosarcoma. The deficiency was determined by an elevated urinary excretion of the histidine catabolite, formiminoglutamic acid, following a loading dose of histidine to the tumor-bearing rats. The folate deficiency of the tumor-bearing rats could not be attributed to altered patterns of dietary consumption or of hepatic histidine-catabolizing enzymes. Further, the oligoglutamate folate fraction of the livers of rats bearing the Novikoff hepatoma was essentially identical to that of normal rats. The elevated formiminoglutamic acid excretion by rats bearing the Novikoff hepatoma could be reversed by high dietary levels of methionine and choline. High dietary levels of folate and of vitamin B_{12}, alone or in combination, had no effect on the elevated formiminoglutamic acid excretion by the hepatoma-bearing rats; similarly, the addition of glycine, serine, formate, thymine, adenine, and guanine to the diets of rats bearing the Novikoff hepatoma did not diminish the elevated urinary formiminoglutamic acid levels. The results indicate that the folate deficiency of tumor-bearing animals may be the consequence of an excessive requirement for methyl groups in the host-tumor system.

INTRODUCTION

Several investigators have demonstrated an elevated excretion of the histidine catabolite, FIGLU,[3] in the urines of patients bearing a variety of tumors (1, 7, 9, 17, 28). Such FIGLU excretion has been correlated with the folate deficiency noted in tumor-bearing patients (2). It has been suggested that the folate deficiency of cancer patients was the result of a high folate requirement by the tumor (2), of inanition (2), or of poor intestinal absorption (28). Except in certain cases of leukemia in which the intracellular folate content of the leukemic cells is exceptionally elevated, none of the postulates appears to have been adequately demonstrated (2, 34).

Urinary excretion of FIGLU has also been found to be elevated in a variety of nutritional and metabolic disorders involving the essential 1-carbon compounds: folate (2), vitamin B_{12} (3), and the methyl donors, methionine and choline (3, 15, 32). In such cases the excess FIGLU probably reflects altered hepatic levels and activity of the folic acid cofactors which are regulated by the nutritional status of the animal with respect to folate (2), vitamin B_{12} (3), choline (8), and methionine (3, 6, 12, 22, 33). It is quite possible, therefore, that the elevated FIGLU excretion of tumor-bearing patients is an indirect consequence of a derangement in the metabolism of methionine, choline, or vitamin B_{12}. In fact, marked abnormalities in the content and metabolism of methionine (5, 14, 29), choline (10, 19, 27), and vitamin B_{12} (11, 18, 21, 24) have been reported in a broad spectrum of experimental tumors. The present studies describe an experimental system used to examine the causes of the folate deficiency observed in tumor-bearing animals. The results provide evidence that an abnormal requirement for methyl donors by the host-tumor system is responsible for this deficiency.

MATERIALS AND METHODS

Compounds. The following compounds were used in these experiments: L-histidine (Eastman Organic Chemicals, Rochester, N. Y.); DL-methionine and folic acid (Matheson, Coleman and Bell, East Rutherford, N. J.); adenine, thymine, and guanine (Nutritional Biochemicals Corp., Cleveland, Ohio); DL-serine, L-urocanic acid, FIGLU, FIGLU enzymes, and tetrahydrofolic acid (Sigma Chemical Co., St. Louis, Mo.); choline chloride and crystalline vitamin B_{12} (General Biochemicals, Chagrin Falls, Ohio); sodium formate (J. T. Baker Chemical Co., Phillipsburg, N. J.), and glycine (Fisher Scientific Co., Montreal, Canada).

Animals and Diets. Young adult male rats (150 to 200 g) were used throughout these experiments. Wistar rats (Canadian Breeders, Inc., St. Constant, Quebec, Canada) were used for the studies involving the Walker 256 carcinosarcoma; Sprague-Dawley Rats (Medico, Inc., Laval, Quebec, Canada) were used in studies with the Novikoff hepatoma. Upon arrival at this laboratory the animals were main-

[1] Supported by grants from the National Cancer Institute of Canada and the Conseil de la Recherche Médicale du Québec.

[2] To whom requests for reprints should be addressed, at National Cancer Institute, the NIH, Bethesda, Md. 20014.

[3] The abbreviations used are: FIGLU, *N*-formimino-L-glutamic acid; histidase, L-histidine ammonia lyase (EC 4.3.1.3.); FIGLU transferase, *N*-formimino-L-glutamate:tetrahydrofolic acid 5-formiminotransferase (EC 2.1.2.5.).

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tained 5 per cage in plastic, wire-screen-topped cages containing wood shavings (Bran de Scie, Montreal, Canada); food (rat chow, Purina Ralston of Canada, Ltd., Woodstock, Ontario, Canada) and water were available *ad libitum*. One week later the rats were given s.c. injections of 5×10^7 ascitic tumor cells and returned to their cages. In experiments involving dietary supplementation with 1-carbon compounds, the pelleted rat chow diet described above was replaced by powdered Micro-mix laboratory chow (Purina Ralston) containing the appropriate level of the 1-carbon compound immediately after tumor transplantation.

FIGLU Analyses. Ten to 14 days after tumor transplantation the animals were weighed and given i.p. injections of L-histidine (225 μmoles/100 g body weight, 225 μmoles/ml 0.89% NaCl solution). The urine samples were collected for 18 hr and the FIGLU analyses were performed as previously described (26). The experiment on the effects of dietary supplementation on FIGLU excretion by Novikoff-hepatoma-bearing rats required large numbers of animals; the experiment was thus subdivided into several smaller ones, each of which had both tumor-free and tumor-bearing controls. The data from all comparable groups was then combined for tabulation. Like other investigators (7, 17), we found that FIGLU excretion values had a positive asymmetrical distribution. Thus, for statistical analysis, the values were normalized by a logarithmic conversion and then reconverted for presentation in the text and Tables (4).

Enzyme Determinations. The hepatic levels of the enzymes histidase, urocanase, and FIGLU transferase were determined in the livers of rats bearing the Novikoff hepatoma, the Walker 256 carcinosarcoma, and in the livers of the corresponding control rats. Ten days after tumor transplantation, the tumor-bearing and the control rats were sacrificed, and their livers were quickly excised and homogenized (26). The assays of histidase, urocanase, and FIGLU transferase were performed as previously described (20, 26).

Folate Determinations. The Novikoff hepatoma was transplanted as described above. Ten days later the animals were sacrificed by decapitation. After exsanguination, the livers and tumors were immediately excised and placed on ice. The grossly viable neoplastic tissue of each tumor was isolated from the necrotic material. The collected tumor samples and livers from each rat were then weighed and homogenized with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) with 9 volumes of ice-cold, 0.1 M phosphate buffer, pH 7.0, containing 8.24 mM ascorbic acid. The homogenates were then placed in a water bath for 5 min at 100° to precipitate the proteins; then the samples were cooled in ice and centrifuged at 2000 rpm for 15 min in an International Model PR-2 refrigerated centrifuge. The supernatants were collected and stored at 5°, and their folate contents were determined (before and after conjugase treatment) with *Lactobacillus casei*, as previously described (36); the oligo- and polyglutamate fractions of tissue folate could thus be separately determined. These assays were generously performed by Dr. V. Michael Whitehead of Montreal General Hospital.

RESULTS

Elevation of FIGLU excretion by Tumor-bearing Rats. Rats bearing the Novikoff hepatoma or the Walker 256 carcinosarcoma excreted elevated quantities of FIGLU into their urines following a loading dose of histidine (Table 1). The elevated FIGLU excretion by both tumor-bearing groups was significant ($p < 0.01$). Sham-operated control rats, given s.c. injections of 1.0 ml of 0.89% NaCl solution instead of tumor cells, excreted normal levels of FIGLU (Table 1); similarly, rats given a loading dose of histidine immediately after transplantation of the Novikoff hepatoma excreted normal amounts of FIGLU in their 18-hr urine sample (Table 1). Thus the elevated FIGLU excretion of the tumor-bearing rats appeared to be dependent upon the growth of the tumor and not upon the transplantation procedure. The elevation in FIGLU excretion observed with the tumor-bearing rats did not appear to be attributable to a decreased food intake by these animals. When the tumor-bearing and the control rats were pair-fed for 10 days, rats bearing the Novikoff hepatoma or the Walker 256 carcinosarcoma still excreted elevated levels of urinary FIGLU (Table 2).

Enzyme and Folate Levels. The possibility that the elevated FIGLU excretion seen with the tumor-bearing animals might be the result of an altered hepatic complement of the enzymes responsible for the biosynthesis and catabolism of FIGLU was investigated. The results are summarized in Table 3. No significant differences could be noted between the levels of histidase, urocanase, and FIGLU transferase noted in the livers of rats bearing the Novikoff hepatoma and the corresponding enzyme levels observed in the livers of control rats. Similarly, the hepatic levels of urocanase were essentially identical in normal rats and in rats bearing the Walker tumor 256. On the other hand, the levels of histidase and FIGLU transferase were significantly lower in the livers of rats bearing the Walker

Table 1

FIGLU excretion in the urines of rats bearing the Novikoff hepatoma and the Walker tumor 256

Ten to 14 days after tumor transplantation the rats were given an i.p. injection of 225 μmoles of L-histidine per 100 g body weight in 0.89% NaCl solution. The FIGLU content of the individual 18-hr urine samples was determined as described in "Materials and Methods."

Group	No. of Animals	FIGLU excreted (μmoles/100 g body wt)		
		Mean	95% confidence Limits	
			Lower	Upper
Control	10	3.2	2.2	4.7
Novikoff	12	13.6	8.9	20.9
Control (sham-operated)	9	3.4	2.2	5.2
Novikoff (0-time)	5	3.1	1.6	6.4
Control	12	3.5	2.8	4.4
Walker tumor 256	10	7.3	3.7	14.6

Table 2

The effect of pair-feeding on the FIGLU excretion in the urines of rats bearing the Novikoff hepatoma and the Walker tumor 256

Ten to 14 days after tumor transplantation, pair-fed rats were given an i.p. injection of 225 μmoles of L-histidine per 100 g body weight in 0.89% NaCl solution. The FIGLU content of the 18-hr urine samples was determined as described in "Materials and Methods."

Group	No. of animals	FIGLU excreted (μmoles/100 g body wt.)		
		Mean	95% confidence Limits	
			Lower	Upper
Control	8	3.7	2.5	5.4
Novikoff	8	12.5	8.9	17.6
Control	8	2.7	1.6	4.6
Walker tumor 256	8	6.8	5.4	8.7

Table 3

The hepatic levels of histidase, urocanase, and FIGLU transferase in normal rats and in rats bearing the Novikoff hepatoma of the Walker tumor 256

The rats were sacrificed 10 days after tumor transplantation and the livers were excised and homogenized in 4 volumes of 0.1 M Tris buffer, pH 8.2. The enzymes were assayed as described (26); each value represents the mean of 6 to 10 determinations.

Group	Enzyme activity (μmoles/hr/g liver)		
	Histidase	Urocanase	FIGLU transferase
Novikoff			
Control	33.8 ± 3.1[a]	30.2 ± 2.3	178 ± 18
Tumor-bearing	28.0 ± 1.7	27.6 ± 1.8	136 ± 15
Walker tumor 256			
Control	22.0 ± 1.0	18.3 ± 1.2	188 ± 7
Tumor-bearing	16.1 ± 1.4	20.2 ± 1.2	146 ± 11

[a] Mean ± S.E.

tumor 256 than they were in the livers of normal rats. However, the differences observed in the hepatic enzyme levels between the normal and the tumor-bearing rats did not appear to be of sufficient magnitude to account for the marked differences in FIGLU excretion. The folate levels observed in the Novikoff hepatoma were found to be much less than those noted in the livers of the control and the tumor-bearing rats (Table 4); the differences were especially striking in the folate fractions that were released by conjugase treatment. The slightly lower hepatic folate levels found in the tumor-bearing rats, compared to those seen in the livers of the tumor-free rats, were found to be of borderline statistical significance ($0.07 > p > 0.06$).

Effects of 1-carbon Compounds on Elevated FIGLU Excretion. The effects of dietary supplementation with essential and nonessential 1-carbon compounds on the elevated FIGLU excretion of rats bearing the Novikoff hepatoma were examined; the results are summarized in Table 5. Only the 2 methyl donors, methionine and choline, reversed the elevated FIGLU excretion of rats bearing the Novikoff hepatoma; the remaining 2 essential 1-carbon compounds, folic acid and vitamin B_{12}, added to the diets of the tumor-bearing rats singly or in combination had no significant effect on FIGLU excretion. None of the nonessential 1-carbon compounds appeared to diminish significantly the elevated FIGLU excretion observed with rats bearing the Novikoff hepatoma (Table 5). Diets containing serine, glycine, formate, adenine, guanine, or thymine did not reduce the elevated FIGLU excretion by the tumor-bearing rats. Thymine, adenine, and glycine appeared to produce significant increases in the FIGLU excretion by the tumor-bearing rats ($p < 0.05$), but the physiological significance of these observations is obscure. Diets containing 0.2% ascorbic acid, which reportedly plays a role in maintaining folic acid activity *in vivo* (16, 31), led to a FIGLU excretion value of 16.6 μmoles/100 g body weight by rats bearing the Novikoff hepatoma; this value is essentially identical to that obtained with the untreated tumor-bearing rats.

DISCUSSION

The present results show a folate deficiency in tumor-bearing rats; this deficiency resembles that very often noted clinically with tumor-bearing patients (1, 7, 9, 17, 28). Previous explanations for the folate deficiency observed with cancer patients have included inanition (2), malabsorption (28), and accumulation of folate metabolites by the tumor (2). The present results, as well as previous work (23, 30) would seem to indicate that none of these mechanisms is sufficiently general to explain the folate deficiency seen with tumor-bearing animals. A more proximate cause of the folate deficiency of the rats bearing the Novikoff hepatoma appears to be an abnormal demand for methyl donors in the host-tumor system. The aberrant metabolism of methyl donors constitutes one of the most common biochemical abnormalities seen in tumors (5, 10, 14, 19, 27, 29). Nutritional deficiences of methyl donors cause a secondary deficiency of folic acid *in vivo* that reflects both an abnormal hepatic content and distribution of the folate cofactors (3, 6, 8, 22, 33); either may be responsible for the physiological symptoms of folate deficiency.

An abnormal distribution of folate cofactors has been proposed as playing a determining role in the cell's choice between differentiation and replication (13, 35). Supporting

Table 4

The folate levels in normal rat liver and in the liver and tumor tissue of rats bearing the Novikoff hepatoma

The rats were sacrificed 10 days after tumor transplantation. The livers and tumors were excised and individually homogenized in 9 volumes of ice-cold 0.1 M phosphate buffer, pH 7.0, containing 8.24 mM ascorbic acid- tissue folate levels were then determined with *L. casei* as described in "Materials and Methods."

Group	Tissue	Tissue folate (μg/g)	
		Without conjugase	With conjugase
Control	Liver	0.33 ± 0.02[a]	25.7 ± 2.4
Tumor-bearing	Liver	0.27 ± 0.04	17.7 ± 2.7
	Hepatoma	0.18 ± 0.04	3.3 ± 0.3

[a] Mean ± S.E. of 4 rats.

Table 5

The effect of dietary supplementation with metabolic 1-carbon compounds on the FIGLU excretion in the urines of rats bearing the Novikoff hepatoma

Immediately after tumor transplantation the rats were placed on diets containing the appropriate 1-carbon supplement; 10 to 14 days later they were given an i.p. injection of 225 μmoles of L-histidine per 100 g body weight in 0.89% NaCl soltuion. The FIGLU content of the 18-hr urine samples was determined as described in "Materials and Methods."

Group	Concentration of supplement in diet (ppm)	No. of animals	FIGLU excreted (μmoles/100 g body wt)		
			Mean	95% confidence limits	
				Lower	Upper
Control		23	2.4	1.7	3.5
Hepatoma		35	9.0	6.5	12.3
+ methionine	15,000	12	1.8	0.6	5.5
+ choline chloride	10,000	11	1.5	0.7	3.1
+ folate	40	5	11.5	5.5	24.0
+ folate	400	7	7.5	3.4	16.3
+ vitamin B_{12}	0.5	14	9.4	5.8	15.2
+ vitamin B_{12}	5.0	9	11.3	8.3	15.6
+ vitamin B_{12} and folate	0.5 40	12	14.0	9.4	20.9
+ glycine	50,000	11	15.0	11.4	19.8
+ serine	50.000	14	16.5	8.3	32.6
+ formate	6,700	9	19.1	11.1	32.8
+ adenine	5,000	10	28.3	27.2	29.5
+ guanine	5,000	5	19.1	9.7	37.0
+ thymine	5,000	6	17.6	11.7	27.1

evidence for this proposal has been the selective toxicity of aminopterin to developing frog embryos (13) and the high ratio of oligoglutamate to polyglutamate forms of folic acid in rapidly dividing tissues noted in the present and previous (26, 35) studies. The established role of methyl donors, both in differentiation (25) and in the control of folate metabolism and distribution (3, 6, 22, 26, 35), is consistent with this hypothesis. Regardless of the mechanism involved, however, the present results indicate that there may be a causal relation between the abnormal methyl metabolism seen in a broad spectrum of tumors (5, 10, 14, 19, 27, 29) and the common clinical observation of folate deficiency in cancer patients (1, 2, 7, 9, 17, 28).

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